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Parasitism of the Zweeloo Woman: Dicrocoeliasis evidenced in a Roman period bog mummy

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Abstract
We undertook the analysis of Zweeloo Woman, a bog mummy from the Netherlands, to assess her parasitic state. Evidence of infection came from two areas: (1) liver paraffin sections and (2) microfossils washed from an intestinal section. Although the liver had shrunken considerably, objects consistent with operculated trematode eggs were found. After evaluating the range of trematode species that produce eggs in liver tissue, we arrived at the diagnosis of *Dicrocoelium dendriticum*. Although only 0.1 ml of sediment was recovered from an intestinal section, eggs of *Ascaris lumbricoides* and *Trichuris trichiura* were also identified. No eggs of *D. dendriticum* were revealed by the intestinal wash although they were observed in the liver. The lancet fluke, *D. dendriticum*, is a zoonosis that usually infects ruminants such as cattle. Eggs of *D. dendriticum* may be found in human coprolites if infected cow liver, for example, was eaten. This is false parasitism. Since eggs of *D. dendriticum* were found in the liver of Zweeloo Woman, we are assured this was a true infection. This find is especially significant because it is the oldest known, patent infection of *D. dendriticum* in humans.

Keywords: parasitology, bog bodies, Zweeloo Woman, mummies, Intestinal wash, *Dicrocoelium dendriticum*

1. Introduction

Since mummies retain the combination of intestinal contents, soft tissue and hard tissue, they have more to reveal about parasitic infection and disease than any other class of archaeological remains. On December 5th, 1951, two peat-cutters found the mummified body of a woman in a bog. The location was known as the "Damsel's Bog", northwest of the villages of Aalden and Zweeloo (Province of Drenthe, the Netherlands). She was named "Zweeloo Woman" after the municipality in which she was found (van Zeist, 1952) (Figure 1).

The Zweeloo Woman was an adult between 35 and 50 years of age at time of death, as assessed by the examination of bones and teeth (Bianucci et al., 2012). Radiocarbon dating performed on both skeletal and skin tissue suggested that Zweeloo Woman lived during the Roman period (average of two radiocarbon dates: 1861 ± 35 BP, calibrated 2σ: 78–233 cal AD) (van der Sanden, 1990). When discovered, she was unclothed and lying on her ventral side with her limbs oriented in a somewhat fetal position. Her remains consist of a nearly complete skeleton, internal organs, and skin.

Zweeloo Woman's place of interment lies within the boundaries of the village municipality (Marke) of Aalden, which dates back to the Middle Ages and probably long before. The eastern boundary is formed by the brook valley of the "Aalder Stroom." The Roman period settlement in which she may have lived has not been discovered. Most likely this settlement is still hidden under the plaggen soil "Aalder Esch," situated to the south of Aalden where an Early Medieval cemetery was excavated. The distance between the bog in which Zweeloo Woman was discovered and her assumed settlement is about 2.5–3 km.

Zweeloo Woman's intestines and other organs were preserved by anaerobic conditions in combination with natural tannic acid in the bog. In bog bodies, liver and kidneys are generally not as well-preserved as skin. These two visceral organs are commonly reduced in size or are unrecognizable due

Figure 1. The location of Zweeloo in the Province of Drenthe, the Netherlands. Courtesy Groningen University, Groningen Institute of Archaeology.

to bog pressure. The lungs and intestinal walls (excluding epithelial lining) are the most commonly preserved and recognizable internal organs (Aufderheide, 2003). Fortunately, Zweeloo Woman's intestine, kidneys and liver were preserved.

2. Materials and methods

The intestine, liver and kidneys were identified by anatomical association and general morphology. Small tissue biopsies (0.5 cm × 0.5 cm) were taken from the liver and kidney. They were analyzed following the methods described in Mekota and Vermehren (2005). After rehydration in Solution III for 48 h, samples were fixed for 24 h in 4% formaldehyde, dehydrated, and finally embedded in paraffin blocks, which were cut on a microtome in 3 μm thick sections (Leica, RM2245). These were histochemically counterstained with either hematoxylin and eosin stain (H&E) or Gram stain (Mulisch and Welsch, 2010).

An intestine section, approximately three centimeters long, was studied. No visible coprolites or other contents were observed in the section. Therefore, we devised a method, not before published, to recover botanical microfossils and parasites eggs. The section was placed in a gridded (1 cm^2) Petri plate and rehydrated using an aqueous solution of 0.5% trisodium phosphate. After treatment, the sample increased approximately 50% in size and regained the appearance and resilience of a fresh intestine section.

The exterior of the rehydrated intestine was washed for microscopic remains. The rehydration fluid from the Petri plate and the wash fluid were centrifuged in a 50 ml centrifuge tube to concentrate potential microscopic remains. This was labeled "exterior wash" and served as a control sample. Then the section was split along the longest dimension and the section was opened. The interior of the section was washed with a jet of distilled water into a clean beaker and gently scraped with a small lab spatula. The fluid from the interior wash was screened through a 250 μm mesh screen to trap macroscopic remains. The fluid that passed through the screen was captured in a 600 ml beaker and was concentrated by centrifugation. To loosen and recover as many microfossils as possible, the open intestine was sonicated for 30 s in a 50 ml centrifuge tube. The intestine was removed from the tube and the microresidues were concentrated by centrifugation. The two tubes of internal microresidues were combined together and labeled "interior wash."

Kumm et al. (2010) applied a newer method of quantifying parasite data based on earlier palynology methods (Maher, 1981; Reinhard et al., 2006). This is the "microfossil concentration method" that allows one to calculate the approximate number of microfossils, including parasites, per unit measure of coprolites by adding known numbers of exotic microfossils. This method can be applied to any other microresidues in archaeological samples. When the microresidues were screened into beakers, we added one tablet of *Lycopodium* (batch 212761, University of Lund, Sweden) containing 12,489 plus or minus 491 *Lycopodium* spores to each beaker. Calculating the numbers of microfossils can be done using the following formula:

$$\frac{(m/l)xt}{x}$$

where m is the microfossils counted; l is the *Lycopodium* spores counted; t is the number of *Lycopodium* spores added to the sample; x is the volume of sample.

Twenty microscope slide preparations of the material from the intestinal contents were made for analysis of potential parasites. Fluid from the exterior wash tube was also studied. Examinations of slides of prepared materials were made with a Zeiss Axiophot microscope using Normarsky imaging and Zeiss Axiovision software.

The concentration of parasites was calculated per ml of sediment based on the above formula.

3. Results

The paraffin sections of the kidney displayed a perfect overview of the kidney tissue including major characteristic regions such as the cortex and the renal pelvis. Additionally, tubule-like structures were identified in the renal corpuscle. Compared to the kidney, the liver was poorly preserved and shrunken. Nevertheless, liver parenchyma with polygonal-shaped hepatocytes and connective tissue could be clearly distinguished. Unfortunately, the loss of other liver-specific structural details and the poor preservation of the overall structure of the tissue material made it impossible to reconstruct the degree of shrinkage of the liver tissue.

Most importantly, operculated, capsule-shaped eggs characteristic of trematodes were visible in the paraffin sections (Figures 2 & 3). Differential diagnosis of the trematode eggs was complicated by shrinkage of the liver. It was further complicated by the absence of free eggs in the analyzed sample from the small intestine. Of the several species of flukes that have been reported from humans, relatively few could potentially occur in humans of the Roman Period in the Netherlands. Flukes of this time and region include *Fasciola hepatica*, *Fascioloides magna*, and *Dicrocoelium dendriticum*. Other common liver flukes of humans are restricted to Asia and Africa. Therefore, in consideration of diagnosis, we excluded *Fasciopsis buski*, *Dicrocoelium hospes*, *Paragonimus westermani*, *Clonorchis sinensis*, *Fasciola gigantica*, or any of the *Schistosoma* species. Thus, the flukes we are considering for diagnosis are *F. hepatica, F. magna*, and *D. dendriticum*.

Morphology comparison between the three trematodes can further identify possible liver flukes of Zweeloo Woman. Both *F. hepatica* and *F. magna* have ellipsoid eggs. The width of the operculum of these species' eggs is less than one-third of the egg width. *D. dendriticum* eggs are capsule-shaped and the operculum is about two thirds of the egg width. The eggs of *F. hepatica* are characterized by eggs ranging in size from 140 to 180 μm. *F. magna* eggs are 180 μm. *D. dendriticum* eggs are 42 by 28 μm. The shrunken eggs exhibited in Zweeloo Woman's liver are 20–25 μm in length. The shape of the eggs in the liver is more consistent with *D. dendriticum*. It is difficult to imagine that shrinkage of Zweeloo Woman's liver could be in an order of 5.6 or 7.2 for *F. hepatica* and *F. magna*, respectively. When we rehydrated the intestinal tissue, it expanded to twice the size, which verifies a remarkable shrinkage of tissue in this mummy. Thus, with regard to morphology, we believe that these trematode eggs are well within the expectation of *D. dendriticum* in shrunken, mummified liver tissue. It is noteworthy that there was a range of preservation exhibited by the eggs in tissue sections.

No *D. dendriticum* eggs were found in the intestinal wash. This may be due to the small amount of microresidues that were recovered. However, other human specific parasites were found in the intestinal wash. Extensive examination of 20 slides revealed traces of *Trichuris trichiura* and *Ascaris lumbricoides* eggs (Figures 4 & 5). Compared to other mummy studies, the eggs of *T. trichiura* and *A. lumbricoides* from Zweeloo Woman are relatively poorly preserved. The mammilated coats of the *Ascaris* eggs are shrunken, and the plugs and larvae of the *Trichuris* eggs have disappeared. For each egg, 95 *Lycopodium* spores were counted. The concentration of eggs for species was calculated to be 13 eggs per ml of sample. No microscopic remains were found in the exterior wash; this confirms the parasite eggs did not enter the intestine through soil contamination.

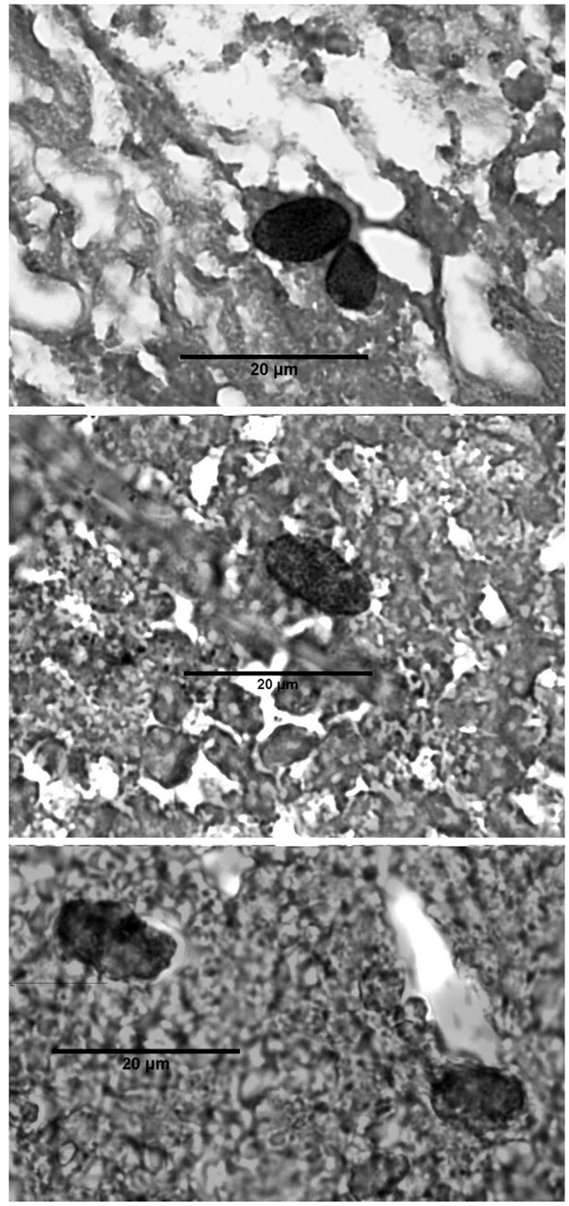

Figure 2. The eggs of *Dicrocoelium dendriticum* were universally shrunken to less than half of their normal dimensions of 40 μm in length by 25 μm in width. Although some eggs were distorted by the shrinkage of surrounding tissues, some retained their distinctive operculated form in gram-stained paraffin sections of tissues. Well-preserved eggs were found liberated from the liver near the kidney (upper image) and in the tissue of the liver (lower image). However, poorly preserved specimens such as shown in the lower image exhibited vaguely recognizable trematode features.

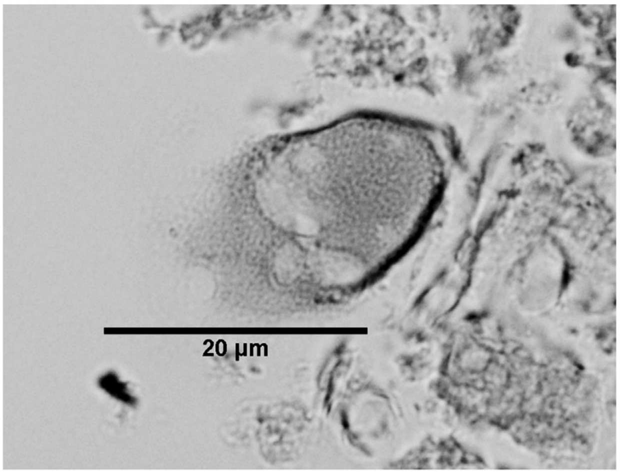

Figure 3. Cross section of a *Dicrocoelium dendriticum* egg showing thick wall and displaced operculum towards the right of the egg.

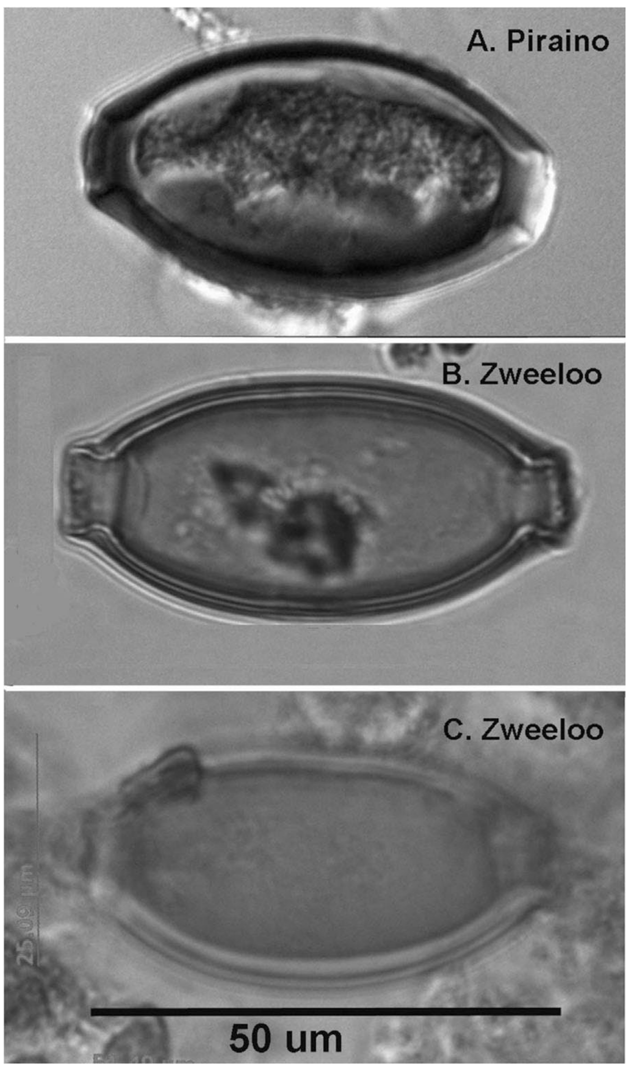

Figure 4. *Trichuris trichiura* eggs from mummies. The egg labeled "Piraino 1" is a well-preserved egg typical of those recovered from desiccated mummies, in this case from Sicily (Kumm et al., 2010). The lower eggs were recovered from Zweeloo Woman. The middle egg shows the absence of an embryo and polar plugs. The lower image shows that the egg shell morphology is well-preserved. This indicates that although the shell preserves well in acidic bogs, the less durable egg components decompose.

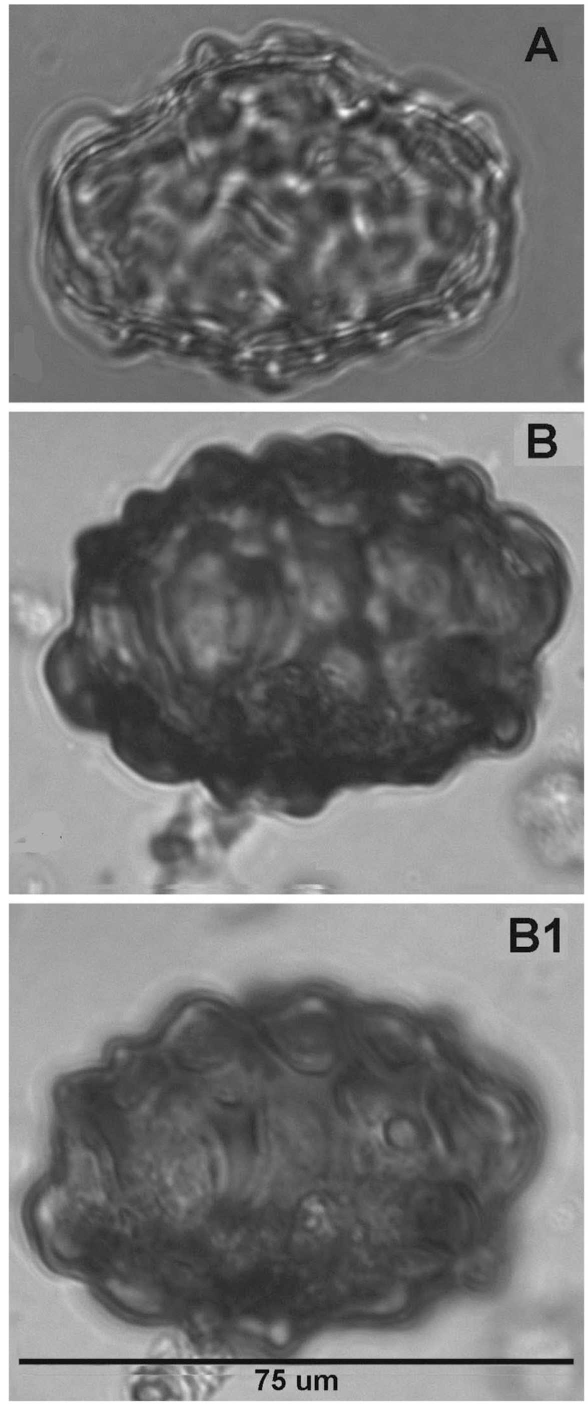

Figure 5. *Ascaris* eggs. The upper image shows the general appearance of an *Ascaris lumbricoides* egg from Zweeloo Woman. The lower images are of a different egg. The shell and mammilated coat preserved well, but no eggs showed preservation of the internal contents.

4. Discussion

The most important discovery by histological analysis was the identification of eggs of *D. dendriticum* embedded in the liver parenchyma. The adult fluke lives in the gallbladder and bile ducts of its herbivorous definitive host. Eggs are passed in the feces of the infected herbivore and are swallowed by the first intermediate host, the land snail. Miracidia emerge inside the snail's intestine and develop into cercariae after approximately three to four months. Cercariae are subsequently excreted into the environment in slime balls which are eaten by ants that represent the second intermediate hosts. Once ingested by ants, the swallowed cercariae develop into metacercariae in the hemocoel of the ant. They become infective to any definitive host that might accidentally ingest ants while grazing. Some metacercariae, however, encyst into the sub-esophageal ganglion of the ant, and they affect the insect's behavior. Instead of going back to the burrows when the daytime temperature decreases, the infected ant moves up to the top of the grass. It clamps its mandibles into the plants and remains paralyzed as long as the temperature remains below 20 °C. As a consequence, the ant becomes accidentally ingested by herbivores and the continued survival of the fluke is ensured. In the duodenum of the final host, metacercariae excyst and develop into flukes, which migrate into the common bile duct. The adult fluke, a hermaphrodite, matures in six to seven weeks and produces eggs, thus completing the life cycle.

False infection in humans is the consequence of eating raw or undercooked animal liver. In this case, eggs pass through the digestive tract unchanged, and if detected, this can result in reported pseudoparasitism (Magi et al., 2009). True human infection is rare and results from the ingestion of ants infected by the larval stage of the parasite. Infected ants may be found in grass, herbs, raw fruit, vegetables and even drinking water. When true human infection occurs the lancet fluke colonizes the bile ducts and the liver. Currently, the lancet liver fluke is widely present in less developed areas of Europe and the Middle East. It is sparsely distributed in Africa, Australia, and the New World (Otranto and Traversa, 2002).

Le Bailly and Bouchet (2010) recently summarized the evidence of *D. dendriticum* in the archaeological record. They emphasize that all finds to date come from coprolites and latrines. These may be reports of false parasitism because eggs from infected liver eaten by humans pass through the intestinal tract in recognizable form. The findings may simply represent dietary behavior (Le Bailly and Bouchet, 2010). In this context, the findings from Zweeloo Woman are very important. Because the eggs were found in the liver of the human mummy, we can be certain this represents a true, patent infection. This evidence, in turn, lends credence that other finds of *Dicrocoelium* sp. parasite eggs in ancient coprolite samples represent a long-term host-parasite evolution as previously suggested (Le Bailly and Bouchet, 2010).

The presence of the lancet fluke in Western Europe was attested from 550,000 years BP (Jouy-Avantin et al., 1999) to historic times (Le Bailly and Bouchet, 2010). There are three published records concerning the Roman Period (1st c. BC to 4th c. AD). The first record was published by Pike (1968). It describes the recovery of *Dicrocoelium* sp. eggs found in cesspit sediments at the Owlesbury site, near Winchester, England (Pike, 1968). The second and the third records are from France; the analysis of cesspit sediments from Reims, and more recently, the investigation of sediments from wood-lined cesspits recovered in Troyes (Le Bailly and Bouchet, 2010) confirmed the presence of the lancet fluke in northeastern France in antiquity, as indicated by zooarchaeological data (Leblay et al., 1997; Lepetz et al., 2002). Its identification in the New World dates back to the 17th century AD following the colonization of Canada by Europeans (Horne and Tuck, 1996). Studying Zweeloo Woman has provided evidence of *D. dendriticum* in the Netherlands. Because the eggs were embedded in the liver parenchyma, this is evidence for the first true record of human dicrocoeliasis.

Previous reports have shown that Zweeloo Woman suffered parasitic infections caused by whipworm (*T. trichiuria*) and giant intestinal roundworm (*A. lumbricoides*) (Paap, 1990). Our analysis confirms the diagnosis and also demonstrates the infections were light. Such light infections of whipworm and roundworm do not cause pathology. Zweeloo Woman evidently did not experience pathological insult from the minimal infections of *A. lumbricoides* and *T. trichiura*. Similarly, infection of *D. dendriticum* generally produces only mild pathological effects. Bloating, diarrhea, and other digestive incidences are most common as a result of the adult fluke existing in bile ducts. In more severe infections, hepatomegaly can result.

It is noteworthy that all examined bog mummies have been infected by parasites. Roundworm and whipworm have been the most commonly discovered. As reviewed by Leles et al. (2010), both roundworm and whipworm eggs were found in the bog mummies of Lindow Man II, Dröbnitz Girl, and Karwinden Man. Whipworm was found in Tollund Man and Grauballe Man (van der Sanden, 1990). Fecal-borne parasitism was ubiquitous in Europe by the Iron Age (Leles et al., 2010). Bog bodies commonly reveal infection. Our recent findings prove these parasites were also present during the Roman Period and, furthermore, provide the first case of true dicrocoeliasis in an individual from the bogs.

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